**Figure legends**

**Fig. 1. Isolation of two distinct populations of *de novo* DNA-containing particles.** (A) Viruses (1010 DNA-containing particles) were taken 8 dpi from the media of infected A9 monolayers with (SN + Cells) or without (SN) repeated freezing and thawing cycles. Anion-exchange chromatography (AEX) was performed and fractions were collected progressively. DNA-containing particles in each fraction were quantified by qPCR. (B) SN virus was treated with 50 U DNaseI prior to AEX. (C) A9 cells (3 × 106) were infected at a MOI of 5000 DNA-containing particles per cell for 1 h at 4 °C, followed by washing to remove unbound virus. The cells were further incubated at 37 °C for 18 h. The nuclei of infected cells were purified and nuclear viruses were extracted as outlined in Materials and Methods. Isolated nuclear progeny was subjected to AEX-qPCR analysis. The infection was performed in the presence of neuraminidase and mouse α-capsid mAb (B7) to avoid re-infections. (D) Empty capsids (EC) were isolated from the SN of infected A9 cells 8 dpi by CsCl gradient centrifugation. Following desalting and resuspension in TE-buffer pH 8 they were subjected to AEX. For analysis of the AEX profile, 2 uL of each fraction were spotted on a nitrocellulose blotting membrane which was probed with B7 α-capsid mAb, followed by a horseradish peroxidase-conjugated secondary antibody.

**Fig. 2. FC-P1 and FC-P2 are infectious but differ in their N-VP2 conformation.** (A)Fractions enriched in FC-P1 or FC-P2 (fractions 10-12 and 14-17, respectively, see Fig. 1A) were pooled, dialyzed in TE-buffer pH 8 and re-subjected to AEX. DNA-containing particles in each fraction were quantified by qPCR. (B) A9 cells (8 × 103) were infected with purified FC-P1 or FC-P2 particles at a MOI of 2500 DNA-containing particles per cell for 1 h at 4 °C, followed by washing to remove unbound virus. The cells were further incubated at 37 °C for 40 min or 22 h. Total DNA was extracted and quantified as described in Materials and Methods. (C) Immunoprecipitation of 108 FC-P1 or FC-P2 particles with a B7 α-capsid mAb (total) or a rabbit α-N-VP2 pAb. Specificity of the antibodies was confirmed using unspecific rabbit IgG. (D) FC-P1 particles (108) were incubated at 50 °C or at pH 4.5. Treated or untreated particles were immunoprecipitated using a B7 α-capsid mAb (total) or α-N-VP2 pAb. Specificity of the antibodies was confirmed using unspecific rabbit IgG. (E) Purified FC-P1 or FC-P2 particles (108) were incubated at pH 7, 6, or 5 and subjected to CHT treatment (+) or not (-). Proteolytic N-VP2 processing was analyzed by 10 % SDS-PAGE. After transfer to a polyvinylidene difluoride membrane, the blot was probed with a rabbit α-VP pAb, followed by a horseradish peroxidase-conjugated secondary antibody. (F) A9 cells (3 × 105) were infected with purified FC-P1 or FC-P2 as indicated above. At different intervals post-infection the proteolytic processing of N-VP2 was examined by immunofluorescence with B7 α-capsid mAb (green) and α-N-VP2 pAb (red). (G) Purified FC-P1 particles (1010) were treated at pH 7 or pH 4.5 followed by dilution in TE-buffer pH 8 and AEX analysis. Eluted fractions were quantified by qPCR.

**Fig. 3. The surface phosphorylation status determines the AEX profiles of FC-P1 and FC-P2.** (A) Nuclear virus progeny (1010 DNA-containing particles, see Fig. 1C) was resuspended in TE-buffer and treated with CHT (0.5 mg/mL) for 1.5 h at 37 °C. Subsequently, the sample was analyzed by AEX-qPCR. (B) Nuclear virus progeny (1010 DNA-containing particles) was treated with lambda phosphatase (40000 U/mL) for 3 h at 37 °C prior to AEX-qPCR analysis. (C) A9 cells (3 × 106) were infected with 5SG mutant viruses as described in Fig. 1C. Nuclear progeny virions (1010 DNA-containing particles) were analyzed by AEX-qPCR analysis. (D) An identical amount of nuclear 5SG progeny virions were treated with lambda phosphatase as outlined above and subjected to AEX-qPCR analysis.

**Fig. 4. FC-P2 progeny actively egresses from the infected host cell.** A9 cells (3 × 106) were infected with 5000 DNA-containing particles per cell at 4 °C. Following washing to remove unbound viruses the cells were incubated at 37 °C in the presence of neuraminidase and B7 for the indicated times. Then, cells were fractionated as explained in Materials and Methods. (A) Progeny in the nuclei of infected A9 cells. (B) Progeny in the cytoplasm of infected A9 cells. (C) Progeny in the media of infected A9 cells. (D) Phase contrast pictures of the infected cells were taken using a Zeiss Axiovert 35 microscope with a 20x magnification objective. Cell viability was accessed via trypan blue exclusion using the TC10TM automated cell counter (BioRad). The average of three independent measurements is indicated.

**Fig. 5. Dynamics of FC-P1 and FC-P2 in infection and transfection.** (A)A9 cells (3 × 106) were infected with 5000 DNA-containing particles per cell at 4 °C. Following washing to remove unbound viruses the cells were incubated at 37 °C in the presence of neuraminidase and B7 for the indicated times. Nuclei isolation and AEX-qPCR analysis were performed at the indicated time points post-infection as specified in Materials and Methods. (B) NB cells (106) were transfected in the presence of neuraminidase and B7 and intracellular virus was immunoprecipitated with B7 mAb and quantified at the indicated time points post-transfection. (C) NB cells were transfected as explained above. AEX-qPCR analysis was performed at the indicated time-points.

**Fig. 6. The phosphoserine-rich N-VP2 is dispensable for active egress.** (A) NB cells (106) were transfected in the presence of neuraminidase and B7. Egressed viruses in the media were quantified following DNaseI treatment. (B) AEX-qPCR analysis of intracellular and released virions was performed 24 hpt and FC-P1 to FC-P2 ratios were calculated.

**Fig. 7. Influence of active egress in infectivity.** FC-P2 particles were purified by AEX from the nuclei of infected A9 cells and from the culture media supernatant. A9 cells (8 × 103) were infected at 4 °C for 1h. Following removal of the unbound virus the cells were incubated at 37 °C for the indicated times. Intracellular DNA was extracted and viral genome copies were quantified.

**Fig. 8. Investigation of the effect of acidic phosphatases on surface phosphorylations during entry.** A9 mouse fibroblasts (3 x 106) were infected with 5000 DNA-containing FC-P2 particles per cell at 4 °C. Following removal of unbound viruses, cells were incubated at 37 °C for the indicated times. In order to inhibit acidic phosphatases, 150 nM BafA1 was added 15 min prior to virus internalization at 37 °C.